0022-1767/93/15[illegible]
The Journal of Immunology

Printed in U.S.A.

Mapping the Major Human T Helper Epitopes of Tetanus Toxin

The Emerging Picture

Jeanette C. Reece, H. Mario Geysen, and Stuart J. Rodda[1]

Chiron Mimotopes Pty. Ltd., Clayton, Victoria 3168, Australia

ABSTRACT. Progress on the mapping of Th epitopes of tetanus toxin (tt) has been slow due to reliance on studies of clones. In this paper, human Th cell epitopes of tt were mapped using proliferation tests on PBMC in response to synthetic peptides. PBMC from nine donors were tested over the entire set of tt homologous overlapping dodecapeptides. The 1304 peptides were initially tested as 66 pools, each containing an average of 20 peptides. PBMC from individual donors responded to as few as 1 and as many as 17 of the 66 peptide pools. The sequences responsible for proliferation were identified for the two most frequently recognized pools, and for another two pools within a major immunodominant region. Three new epitope sequences were mapped in detail and based on their recognition by most individuals are likely to be promiscuous. A cocktail of peptides including the newly identified Th cell epitopes was able to induce proliferation in PBMC from 24 of 31 tetanus toxoid (TT)-responsive donors. This cocktail is a chemically defined reagent that can be used to quantitate in vitro Ag-specific Th cells in PBMC from most subjects, and may thus be useful for serial measurements of specific immunity such as in subjects undergoing immunotherapy or immunosuppressive treatment. *Journal of Immunology*, 1993, 151: 6175.

TT[2] is commonly used in clinical or research studies of human T cell responsiveness as a control Ag or as a model Ag for studying Ag processing, presentation, and recognition mechanisms (1). Known epitopes of the untoxoided protein, tt, have been established by a combination of screening and predictive methods, largely by study of Th clones (2). A limitation of the methods used for initial location of determinant regions was that they relied on efficient processing of protein fragments by pathways similar to those operating with the whole Ag (1). It has been shown that cells deficient in specific enzymes can fail to process Ag and present a particular peptide, despite normal ability to process and present other peptides (3). Thus, use of long peptides or partially fragmented Ag could fail to reveal immunodominant regions of the Ag.

Frequencies of Th cells specific for TT can be very high in PBMC (4). It is therefore feasible to detect individual epitopes by direct stimulation of PBMC with peptides representing single epitopes, because precursor Th cells specific for single epitopes will be present in replicate samples of a donor's PBMC. We have found that use of pools of short synthetic peptides as Ag (J.C. Reece et al., manuscript in preparation) can allow epitope mapping with PBMC of any Ag of known sequence to which humans or animals have a strong Th response.

PBMC from donors shown to respond to TT in vitro were screened against peptide pools to locate the major epitopes in the sequence. The data obtained revealed five major epitopes, of which three had not been reported from studies using other approaches. The epitopes were then used, along with epitopes from other sources, in a survey of unselected donors to look for the breadth of recognition over a range of HLA types. The use of these materials as a chemically defined Ag for quantitation of Th cell responses in a clinical setting is proposed.

Received for publication May 10, 1993. Accepted for publication August 20, 1993.

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[1] Address correspondence and reprint requests to Dr. Stuart J. Rodda, Chiron Mimotopes Pty. Ltd., 11 Duerdin Street, Clayton, Victoria 3168, Australia.

[2] Abbreviations used in this paper: TT, tetanus toxoid; tt, tetanus toxin; b-dkp, beta-amino-alanine-diketopiperazine.

Materials and Methods

Medium

Complete medium consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 5 mM HEPES buffer, pH 7.4, and

20 μg/ml gentamicin (CSL, Melbourne, Australia) to which 10% (vol/vol) human serum, pooled from donations screened for suitability in supporting in vitro PBMC proliferation, had been added.

Ag

Overlapping dodecapeptides for epitope scanning were synthesized by the multipin method (5) with a COOH-terminal b-dkp group and an acetylated NH_2-terminus. NH_2- and COOH-terminal-blocked peptides are as efficient in activation of Th cell clones as unblocked peptides (6, 7), in contrast to cytotoxic T cells (8). Peptides were cleaved into 0.1 M sodium bicarbonate in 96-well microtiter trays. The purity of representative peptides was assessed using HPLC and was found to be generally >80%. Wells were found to contain an average of 10 nmol cleaved peptide by amino acid analysis. Two independently synthesized sets of peptides made on pins were used for the final identification of T cell stimulatory dodecapeptides.

Bulk peptides for testing larger numbers of donors (Table VII) were prepared by solid phase peptide synthesis using an Applied Biosystems 430A peptide synthesizer. Peptides were purified to >90% and their compositions were confirmed by amino acid analysis.

A cocktail of tt epitopes was prepared from equimolar amounts of peptides tt 141–171, 257–268, 591–602, 616–631, 640–651, 652–663, and 947–967. TT was a gift from the Commonwealth Serum Laboratories, Melbourne, Australia.

Cell preparation

PBMC were from anticoagulated venous blood of healthy volunteers. PBMC were isolated by density interface centrifugation over Ficoll-Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The average yield of PBMC from whole blood was 2×10^6/ml with a range of 1.2×10^6/ml to 2.9×10^6/ml.

Standard PBMC proliferation assay

Peptide-stimulated proliferation assays using 2×10^5 PBMC per well were performed in 96-well round bottom microtiter plates (Nunc, Roskilde, Denmark). Ag were added in 20 μl of 0.1 M sodium bicarbonate to PBMC in complete medium to give a final volume of 200 μl per well. Assays were conducted using at least 16 replicates per test group. Cultures were incubated at 37°C in 5% CO_2 in humidified air. After 138 ± 2 h, proliferation was detected by pulsing with 0.5 μCi tritiated [*methyl*-^{3}H]thymidine (40 to 60 Ci/mmol, Amersham Australia, Sydney) per well for 6 h. Cells were harvested onto glass fiber filter mats (Skatron, Sterling, VA), and incorporated thymidine was measured using an LKB 1205 Betaplate liquid scintillation counter. All assays included at least 24 wells each of negative controls (20 μl of 0.1 M sodium bicarbonate instead of peptide solution) and positive controls (TT at 1.0 Lf/ml or 0.1 Lf/ml, also in 0.1 M sodium bicarbonate buffer).

MHC class II typing

MHC typing was performed on whole blood samples or EBV-transformed B cells by the Red Cross Blood Bank, Melbourne.

Method of statistical treatment of results

Data from large numbers of replicates per Ag-stimulated test group clearly demonstrated that the cpm values within a group are not normally distributed. This is a consequence of the random distribution of low numbers of specific responding T cells among the replicate wells. It is therefore inappropriate to treat proliferation data on PBMC by statistical methods based on normally distributed data. We have found the Poisson model is a better representation of the data (H.M. Geysen et al., manuscript in preparation) and therefore chose to use the following method. A cutoff was calculated in the conventional way assuming that data from the unstimulated (cells alone or no Ag negative control) group was normally distributed. A cutoff cpm value of the mean plus three times the SD of the cells alone group was calculated and used to score each well as negative (below the cutoff) or positive. Poisson statistics were used to determine whether any difference in the numbers of positive wells between the negative control (cells alone) group and each experimental group was significant. Frequencies of positive responses significant at the 0.25% or better ($p < 0.0025$) level are reported.

Because this method of analysis is uncommon for proliferation tests but common in other quantal methods, we have included a typical set of data from the pools scan of one donor comparing this method of analysis with a conventional method using the mean ± SD of the ^{3}H-TdR uptake (cpm) (Fig. 1). Figure 1*A* shows that for several peptide tests, the mean is higher than the mean of the cells alone but the SD is generally large so a simple statistical test will not distinguish any test groups from the negative control group. This is a direct result of the low frequency of peptide-specific Th cells: only wells with peptide-specific Th cells can show proliferation. In contrast (Fig. 1*B*). classifying each well as either proliferating or non-proliferating and using a statistical test to distinguish groups significantly different from the cells alone according to the frequency of wells displaying proliferation is logical and objective. Figure 1 also gives an indication of the magnitude of peptide responses and generally shows the higher the mean, the greater the frequency of positive wells, as expected. We assigned a cutoff of the mean + 3SD based on the assumption of normally distributed background cpm, and it is evident that there are borderline cases between

proliferating wells and nonproliferating wells (Fig. 1). This is inevitable where there is a continuous spectrum of values.

Results

Peptide pooling strategy

A set of 1304 overlapping 12mer peptides was synthesized spanning the 1315 residues of the tt sequence (9), each peptide offset by one residue from the preceding peptide. Thus, each peptide overlapped the preceding and the following one by 11 residues. The multipin peptide synthesis system used gave nontoxic peptide solutions ready for use in bioassays. Because it was impractical to screen each peptide separately on each donor's PBMC for its ability to cause proliferation, we used a peptide pooling strategy to identify regions containing Th cell epitopes, followed by testing of individual peptides from the most frequently recognized pools.

We chose to screen peptides as 66 pools of approximately 20 sequential overlapping peptides each (Table I). The size of the pools was selected so that the size of both the initial scan and the subsequent decodes of stimulatory pools would be manageable. Due to the completeness of the peptide set, the peptides from the NH_2-terminal end of a pool overlap with the preceding pool and likewise the peptides from the COOH-terminal end of a pool overlap with the following pool.

The concentration of each peptide used in the final culture was 0.3 μM. Epitopes of less than 12 residues in length will be present in two, three, or more of the overlapping peptides in that pool, and therefore the concentration of shorter epitopes will be higher than that of longer epitopes.

PBMC from nine HLA-typed donors (Table II), known to respond to TT in vitro, were initially scanned for their ability to respond to each of the 66 peptide pools (Table I). Peptide pool/donor combinations scored as positive are those in which proliferation occurred in a significantly larger number of wells than seen in the cells alone control ($p < 0.0025$). Figure 1 shows a typical set of data from the pool scan of one donor comparing this method of analysis with the conventional method of using the mean ± SD.

Table I shows that several pools stimulated PBMC from

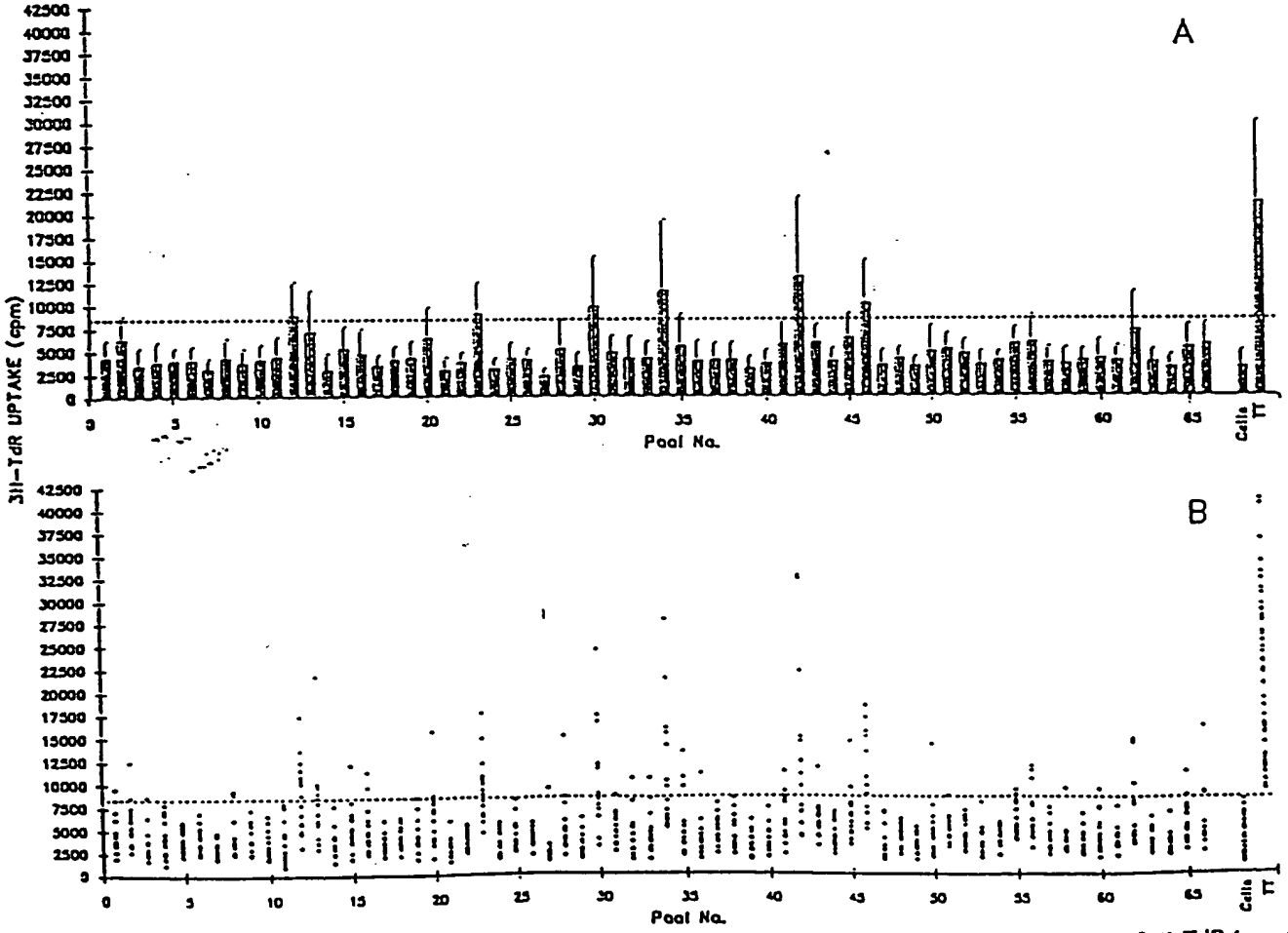

FIGURE 1. The data from the tt pools scan using donor H from Table I. *A*: The mean ± SD of incorporated [^{3}H]-TdR (cpm). *B*: A scatter plot of the incorporated [^{3}H]-TdR (cpm) of individual wells.

Table 1
Complete scan of tt for Th cell epitopes using peptide pools and PBMC

Pool[a] No.	tt Sequence Spanned by Pool	Donor A	B	C	D	E	F	G	H	I	No. +VE Donors
1	1 to 31							+[b]			+
2	21 to 51										
3*	41 to 69										
4	59 to 89										+
5	79 to 109	+									
6	99 to 129							+			+
7	119 to 149					+	+			+	+++
8	139 to 169						+			+	++
9	159 to 189							+		+	++
10	179 to 209		+			+			+		++
11	199 to 229										
12	219 to 249										
13	239 to 269										
14	259 to 289										+
15	279 to 309										
16	299 to 329										
17	319 to 349										
18	339 to 369										
19	359 to 389										
20	379 to 409										
21	399 to 429	+									
22	419 to 449							+			+
23	439 to 469										
24	459 to 489										
25	479 to 509										
26	499 to 529					+		+			+
27	519 to 549										
28	539 to 569					+					+
29	559 to 589										
(30)	579 to 609		+			+	+		+	+	+++++
31	599 to 629		+		+						+
32	619 to 649					+*				+	+
33	639 to 669					+	+			+	+++
34	659 to 689		+						+		++
35	679 to 709								+		+
36	699 to 729										
37	719 to 749			+			+			+	+++
38	739 to 769									+	+
39	759 to 789										
40	779 to 809										
41	799 to 829		+							+	++
(42)*	819 to 847					+	+		+	+	++++
43	837 to 867		+				+				++
44	857 to 887										
45	877 to 907										
46	897 to 927								+	+	++
47	917 to 947										
48	937 to 967										
49	957 to 987						+			+	++
50	977 to 1007						+				+
51	997 to 1027										
52	1017 to 1047										
53	1037 to 1067										
54	1057 to 1087										
55	1077 to 1107										
56	1097 to 1127						+		+	+	+++
57	1117 to 1147										
58	1137 to 1167						+				+
59	1157 to 1187						+			+	++
60	1177 to 1207					+	+			+	+++
61	1197 to 1227										
62	1217 to 1247		+				+		+		+++
63	1237 to 1267									+	+
64	1257 to 1287					+					+
65	1277 to 1307										
66*	1297 to 1315		+					+	+		+++
Negative control		1/112	3/112	0/112	1/80	1/112	3/112	3/112	1/112	2/112	
Positive control (TT)		49/56	38/56	26/56	25/40	14/56	16/56	44/56	56/56	56/56	

[a] Each peptide pool consisted of 20 overlapping 12mer peptides unless specified by an *.
[b] Pools scored positive ($p < 0.0025$) using 16 replicates per test.

Table II
Map of class II MHC Ag for donors in pooling scan of tt

Donor	HLA Typing					
	DR	DR	DRW	DRW	DQ	DQ
	11	13	52		1	7
B	2	4		53	1	7
C	4	7		53	7	9
D	4	7		53	2	8
E	2	3	52		1	2
F	2				1	
G	4	13	52	53	1	8
H	1	7		53	1	9
I	1	2			1	

re than one donor. Two major areas of reactivity were ools 30 (tt residues 579–609) and 42 (tt residues 819–847). A further eight pools stimulated PBMC from one-third (3/9) of the donors. Six of the nine donors responded to a pool unique for that donor, whereas 29 of 66 (44%) of the pools were not shown to be stimulatory for any of the donors tested under the stringent criterion used ($p < 0.0025$).

Location of Th cell epitopes within stimulatory pools

The individual peptides within four stimulatory pools were tested to identify the peptide(s) responsible for proliferative responses incurred by the pool. For convenience, we call this test a decode. Single peptides were tested at 1 μM, approximately three times the concentration of individual peptides used in the pool. This was because when more than one peptide within a pool contains an epitope, the effective concentration is proportionally higher. For example, if an epitope for a single Th cell consisted of nine amino acids (10, 11), stimulatory sequences would be present in four consecutive overlapping peptides within the pool, making the effective concentration of that epitope 1.2 μM.

Decoding of the most commonly recognized pools, 30 and 42 (Tables III and IV, respectively), enabled us to see whether published Th cell epitopes would be precisely identified using this method. Peptides within pool 30 contain sequence YSYFPSVI (tt 593–600), the epitope for a human tt-specific Th cell clone (10). Decoding of pool 30 sh wed that five overlapping 12mers with start residues 589 to 593 were stimulatory for at least one of the three donors (Table III). These 12mers all contain the sequence YSYFPSVI, identical to the published epitope (10, 12).

Pool 42 spans sequence QYIKANSKFIGITEL (tt 830–844), reported to contain a universally immunogenic DR-restricted epitope (1). Decoding of pool 42 showed that five 12mers with start residues between 827 to 831 were capable of stimulating PBMC from four donors tested (Table IV). All these peptides overlap a core (6) of eight residues, YIKANSKF, within the reported epitope tt 830–844 (1).

Because the region tt 579–689 (pools 30 to 34) consisted of five commonly stimulatory pools, we chose to decode two additional poo within this region to identify epitopes not previously reported. Testing of individual peptides within pool 33 on four donors' cells showed that the response to this pool was due to two distinct determinant regions (Table V). Donor B, although not scored positive for this pool in Table I, had shown responses to pool 33 at the less stringent level of $p < 0.05$, and was thus included in the testing on single peptides of pool 33 (Table V). The Th cell epitopes within this region were centered on sequences IVPYIGPA (tt 642–649) and KQGYEGNFI (tt 654–662), respectively.

Decoding of pool 31 and the first two peptides of pool 32 revealed with donor D a series of six overlapping stimulatory 12mers with start residues 616 to 620 (Table VI). All these peptides contain the 7mer core sequence IDDFTNE (tt 620–626). Donors B and G responded to one and two peptides, respectively, containing the core sequence (Table VI). Donor G was included in the testing of single peptides within pool 31 because in the tt pools scan positive responses to pool 31 were significant at the $p < 0.05$ level.

We sought to determine whether these findings using 12mer peptides would also apply for longer peptides. We tested the ability of a 16mer, which encompassed the "envelope" sequence (6) of the stimulatory peptides from pool 31, to stimulate PBMC of donors B and D and a random set of donors (Table VII) (residues 616 to 631). More than half of the donors responded to this 16mer, implying that it is a "promiscuous" epitope.

The other two newly identified T cell determinants (Tables V and VI) and four other peptides containing T cell epitopes of tt were also tested on the random set of donors (see footnote to Table VII). Of the four other tt peptides, two were identified in experiments conducted concurrently with the work reported herein, i.e., tt 141–171, corresponding closely to pool 8 (Table I), and tt 257–268 which was found using an unpublished algorithm (data not shown) but was associated with only two responders in the original scan of nine donors (Table I, pool 13).

In the survey of 32 additional donors (Table VII), peptides were tested at two concentrations, 10 and 1 μM, using 32 replicates per test. The 31-residue tt 141–171 and the 12mer tt 640–651 (Table VII) gave highly significant ($p < 0.0025$) responses at one or both peptide concentrations in at least half of the donors. All of the remaining peptides, including the promiscuous epitope tt 947–967 (1), stimulated PBMC of at least one quarter of the donors. One donor who did not respond to TT in vitro also failed to respond to any of the tt peptides, despite being responsive to other peptide and control Ag (data not shown). This suggests Ag specificity of the responses to tt peptides, which was also suggested in restimulation experiments and studies on immunization of volunteers with TT (J.C. Reece et al., manuscript in preparation).

Three donors responded well to TT but not to any of the

Table III
Decode of stimulatory pool 30 spanning residues of tt 579–609

Peptide Start No.	Sequence	Donor B	Donor F	Donor I
579	TNSVDDALINST[a]	—	—	—
580	NSVDDALINSTK	—	—	—
581	SVDDALINSTKI	—	—	—
582	VDDALINSTKIY	—	—	—
583	DDALINSTKIYS	—	—	—
584	DALINSTKIYSY	—	—	—
585	ALINSTKIYSYF	—	—	—
586	LINSTKIYSYFP	—	—	—
587	INSTKIYSYFPS	—	—	—
588	NSTKIYSYFPSV	—	—	—
589	STKIYSYFPSVI	—	—	3[b]
590	TKIYSYFPSVIS	4	3	—
591	KIYSYFPSVISK	—	3	5
592	IYSYFPSVISKV	4	3	4
593	YSYFPSVISKVN	—	4	—
594	SYFPSVISKVNQ	—	—	—
595	YFPSVISKVNQG	—	—	—
596	FPSVISKVNQGA	—	—	—
597	PSVISKVNQGAQ	—	—	—
598	SVISKVNQGAQG	—	—	—
Cells alone		1/72[c]	2/96	3/120
Pool 30		5/16	6/16	5/16
TT 0.1 Lf/ml		36/40	34/56	55/56

[a] Individual peptides were tested at a concentration of 1 μM.
[b] Number of positive wells out of 16 replicate wells. Only frequencies of positive wells that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from the cells alone control ($p > 0.0025$).
[c] Number of positive wells out of the number of wells shown.

peptides. These results indicate that these tt T cell epitopes display at least partial MHC class II restriction (Table VII). These results also show that the peptides do not exhibit nonspecific mitogenic activity.

Figure 2 summarizes the major human Th cell epitopes of tt, both from this study and from published data, with emphasis placed on those epitopes known or likely to be promiscuous.

Testing a cocktail of dominant human tt Th cell epitopes

To determine whether a cocktail of dominant epitopes of an Ag could be used as a chemically defined reagent in place of the whole Ag, seven Th cell epitopes of tt were pooled together and tested in parallel with TT (Table VII). The cocktail comprised previously reported epitopes (1, 12) and epitopes identified by this study (*Materials and Methods*). The seven tt peptides were tested individually to identify the peptide(s) responsible for responses incurred by the cocktail (Table VII).

As expected, the frequency of positive wells was generally as high as the strongest of the individual peptide frequencies (Table VII). A higher proportion of TT-immune donors (24 of 31) responded to the pool than to any individual peptide. These results confirm that the cocktail used does not have an epitope for all donors, but show that combining epitopes is a practical way to create a chemically defined T cell stimulatory reagent for studies on PBMC.

Discussion

Many Th cell determinant regions of tt were identified, and four of these were examined in detail, resulting in th mapping of a total of five epitopes. The most frequently recognized sequence corresponds to a published Th epitope for a single human T cell clone (10, 12), whereas another corresponds to a published promiscuous Th cell epitope (1). Reliance on predictive methods or on screening of T cell clones for epitope specificity had not previously identified three of these epitopes. These results allow a map of human Th cell tt epitopes to be drawn (Fig. 2). There are clearly further sites to be decoded in detail (Table I) to build up a more complete map.

The success of this approach in identifying epitopes with PBMC may stem from the use of short peptides. Protein cleavage fragments (2) or long synthetic peptides with small overlaps (14) may fail to stimulate Th cells to proliferate, due to cleavage of epitope sites or inappropriate processing of peptide (J.C. Reece et al., manuscript in preparation). Use of all overlapping peptides of a length within the range of naturally processed peptides (13 to 18 residues) (15, 16) can result in presentation of the specified epitope without the need for processing. With the pooling/

Table IV
Decode of stimulatory pool 42 spanning residues of tt 819–847

Peptide Start No.	Sequence	Donor E	Donor F	Donor H	Donor I
819	EFDTQSKNILMQ[a]	—	—	—	—
820	FDTQSKNILMQY	—	—	—	—
821	DTQSKNILMQYI	—	—	—	—
822	TQSKNILMQYIK	—	—	—	—
823	QSKNILMQYIKA	—	—	—	—
824	SKNILMQYIKAN	—	—	—	—
825	KNILMQYIKANS	—	—	—	—
826	NILMQYIKANSK	—	—	—	—
827	ILMQYIKANSKF	7[b]	—	—	—
828	LMQYIKANSKFI	6	—	6	5
829	MQYIKANSKFIG	5	—	14	—
830	QYIKANSKFIGI	4	—	5	—
831	YIKANSKFIGIT	—	3	—	—
832	IKANSKFIGITE	—	—	—	—
833	KANSKFIGITEL	—	—	—	—
834	ANSKFIGITELK	—	—	—	—
835	NSKFIGITELKK	—	—	—	—
836	SKFIGITELKKL	—	—	—	—
Cells alone		2/88[c]	8/252	3/100	1/60
Pool 42		5/24	[d]	4/24	*
TT 0.1 Lf/ml		24/24	24/24	24/24	16/16

[a] Individual peptides were tested at a concentration of 1 μM.
[b] Number of positive wells out of 24 replicate wells. Only frequencies of positive wells that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from the cells alone control ($p > 0.0025$).
[c] Number of positive wells out of the number of wells shown.
[d] Donors F and I were shown to respond to pool 42 in the initial pool scanning assay (Table I).

Table V
Decode of stimulatory pool 33 and the last peptide of pool 32

Peptide Start No.	Sequence	Donor B	Donor E	Donor F	Donor I
638	QVSTIVPYIGPA[a]	•[b]	•	8[c]	—
639	VSTIVPYIGPAL	7	4	•	•
640	STIVPYIGPALN	4	4	•	•
641	TIVPYIGPALNI	6	3	15	10
642	IVPYIGPALNIV	—	—	4	3
643	VPYIGPALNIVK	—	—	—	—
644	PYIGPALNIVKQ	—	—	—	—
645	YIGPALNIVKQG	—	—	—	—
646	IGPALNIVKQGY	—	—	—	—
647	GPALNIVKQGYE	—	—	—	—
648	PALNIVKQGYEG	—	—	—	—
649	ALNIVKQGYEGN	—	—	—	—
650	LNIVKQGYEGNF	—	—	—	—
651	NIVKQGYEGNFI	—	—	10	—
652	IVKQGYEGNFIG	8	2	—	—
653	VKQGYEGNFIGA	—	—	—	—
654	KQGYEGNFIGAL	—	—	—	—
655	QGYEGNFIGALE	—	—	—	—
656	GYEGNFIGALET	—	—	—	—
657	YEGNFIGALETT	—	—	—	—
658	EGNFIGALETTG	—	—	—	—
Cells alone		1/63[d]	3/132	2/77	2/77
Pool 33		23/24	16/24	24/24	16/24
TT 0.1 Lf/ml		23/24	13/24	19/24	24/24

[a] Individual peptides were tested at a concentration of 1 μM.
[b] Not tested.
[c] Number of positive wells out of 16 replicate wells. Only frequencies that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from cells alone ($p > 0.0025$).
[d] Number of positive wells out of the number of wells shown.

p638 - c33
DVSTIVPYIGPALNIV

decoding approach, the otherwise daunting task of testing all these short peptides of an Ag on PBMC of individual donors is achievable.

We have found that the peptide pooling strategy using human PBMC works well for identifying the Th cell epitopes within Ag from influenza, allergens, and HIV-1 (data not shown). The frequencies of Ag-specific Th cells for some of these Ag were generally lower than for TT indicating that it is not necessary to choose Ag with exceptionally high frequencies of Ag-specific Th cells. In addition, we have found that the peptide pooling strategy can be applied to epitope mapping with spleen and lymph node cells from animals as well as to PBMC (data not shown).

The physical length of the peptides used herein is consistent with the 13 to 18 residue length range of the peptides naturally bound to class II Ag (15, 16). This is because all peptides used in the pools contain 12 residues of the tt sequence with a constant tripeptide moiety (b-dkp) (5) at the COOH-terminal end and an acetyl group at the NH_2-terminal end. An acetylated NH_2-terminus can lead to increased effectiveness of Th epitope peptides (7). Th cell clones can be stimulated by b-dkp-bearing peptides f 8, 9, or 10 residues (10) suggesting that peptides containing 12 residues of the Ag sequence have more than the required amount of sequence ed to allow MHC class II binding and recognition by the TCR.

Even though the 12mers detected many previously unknown epitopes, had we used longer peptides we may have detected more determinant regions. For example, individuals frequently respond to tt 947–967 (Table VII) but not to shorter peptides spanning this region (Table I, and additional data not shown). Thus, this new map, although more thorough than any previously reported, is only a first step toward the full set of epitopes for tt. The donors also represent a limited spectrum of MHC types, ensuring that there are further epitopes presented by other allotypes yet to be defined.

Within the four pools decoded, there were cases where at least six overlapping 12mer peptides were stimulatory. The proliferative response to these related peptides could be due to the activation of clonal progeny of one precursor T cell by a sequence common to the peptides. Alternatively, these observations may result from activation of a number of independent T cell clones able to respond to different but overlapping sequences. The reported finding that the NH_2-terminus of the peptide was an important and consistent part of the peptide that binds to MHC class II Ag (15) suggested

Table VI
Decode of pool 31 and the first two peptides of pool 32

Peptide Start No.	Sequence	Donor B	Donor D	Donor G[a]
599	VISKVNQGAQGI[b]	—	—	—
600	ISKVNQGAQGIL	—	—	—
601	SKVNQGAQGILF	—	—	—
602	KVNQGAQGILFL	—	—	—
603	VNQGAQGILFLQ	—	—	—
604	NQGAQGILFLQW	—	—	—
605	QGAQGILFLQWV	—	—	—
606	GAQGILFLQWVR	—	—	—
607	AQGILFLQWVRD	—	—	—
608	QGILFLQWVRDI	—	—	—
609	GILFLQWVRDII	—	—	—
610	ILFLQWVRDIID	—	—	—
611	LFLQWVRDIIDD	—	—	—
612	FLQWVRDIIDDF	—	—	—
613	LQWVRDIIDDFT	—	—	—
614	QWVRDIIDDFTN	—	—	—
615	WVRDIIDDFTNE	—	8[c]	—
616	VRDIIDDFTNES	—	8	—
617	RDIIDDFTNESS	—	8	2
618	DIIDDFTNESSQ	—	12	••
619	IIDDFTNESSQK	7	15	3
620	IDDFTNESSQKT	—	4	—
Cells alone		1/108[d]	2/104	5/216
Pool 31		6/24	16/24	3/24
TT 0.1 Lf/ml		19/20	24/24	24/24

[a] Donor G gave significant proliferative responses at the $p < 0.05$ level.
[b] Individual peptides were tested at a concentration of 1 μM.
[c] Number of positive wells out of 24 replicate wells. Only frequencies of positive wells that were significantly higher than the cells alone control ($p < 0.0025$) are shown. "—" indicates not significantly different from the cells alone control ($p > 0.0025$).
[d] Not tested.
[e] Number of positive wells out of the number of wells shown.

p615 - 631 = WVRDIIDDFTNESSQKT

Table VII
Summary of PBMC responses to tt peptides and peptide cocktail

Donor	Peptide														TT 0.5 Lf/ml	Cells Alone
	141–171[a]		257–268[b]		591–602[c]		616–631[d]		640–651[e]		652–663[e]		947–967[f] 1 μM[h]	Cocktail[g] 1 μM		
	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM				
B	NT	NT[i]	NT	NT	NT	NT	9	3[j]	NT	NT	NT	NT	NT	NT	24/24	0/48
D	NT	NT	NT	NT	NT	NT	29	21	NT	NT	NT	NT	NT	NT	24/24	5/82
CM1	32	32	31	25	4	27	13	5	32	32	7	6	23	32	24/24	1/102
CM2	—	—[k]	—	—	—	—	16	17	—	—	8	—	—	24	24/24	3/102
CM3	—	—	32	32	30	19	28	—	29	—	16	11	5	32	24/24	2/95
CM4	—	—	—	—	—	—	—	—	—	—	—	—	—	8	24/24	2/102
CM5	7	9	17	14	—	—	—	—	17	10	17	—	—	31	24/24	1/102
CM6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	18/18	1/45
CM7	—	—	8	7	—	—	4	—	7	—	4	—	28	31	24/24	1/102
CM8	30	32	—	—	15	—	4	—	24	15	—	—	9	31	24/24	1/102
CM9	13	12	—	—	—	—	—	—	—	—	—	—	—	11	23/24	1/32
CM10	14	16	10	16	NT	NT	19	14	NT	NT	24	NT	7	NT	16/18	0/74
CM11	—	—	28	25	27	23	15	—	10	—	25	10	—	32	24/24	2/140
CM12	—	—	—	—	—	—	—	—	24	—	5	—	—	—	18/18	1/68
CM13	—	—	—	—	6	—	—	—	—	—	—	—	—	26	24/24	2/102
RX45	—	—	—	—	—	—	29	32	10	—	—	—	—	29	24/24	2/102
RX46	31	32	14	22	32	25	32	32	32	32	32	20	31	32	24/24	1/102
RX47	22	8	—	—	—	—	14	8	23	9	—	—	—	20	NT	1/36
RX48	—	—	9	14	13	15	13	5	5	—	10	5	10	28	24/24	2/198
RX49	—	—	—	—	5	—	27	28	5	—	—	—	—	21	15/15	0/38
RX50	9	5	—	—	—	—	—	—	7	—	—	—	—	10	17/24	4/152
RX51	29	18	7	—	14	—	7	—	32	25	18	9	8	26	24/24	2/101
RX53	20	27	—	—	—	7	25	29	24	29	6	5	7	31	24/24	2/140
RX54	8	15	—	—	—	—	—	—	4	8	—	—	4	16	8/24	3/140
RX55	6	3	5	4	—	—	3	—	—	—	—	—	3	15	24/24	0/102
RX56	31	17	—	—	—	—	—	—	3	—	—	—	—	32	24/24	0/102
RX57	6	—	—	—	—	—	—	—	—	—	—	—	—	—	10/24	0/98
RX59	—	—	—	—	—	—	—	—	—	—	—	—	—	—	18/24	2/102
RX60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	24/24	2/102
RX61	13	7	4	5	—	—	—	—	8	7	12	—	—	17	22/24	1/102
RX62	—	—	—	—	—	—	—	—	—	—	—	—	6	—	24/24	1/102
RX63	—	—	—	—	—	—	—	—	—	—	—	8	—	—	23/24	3/102
RX64	—	—	7	—	—	—	13	—	—	—	—	—	—	30	24/24	1/102
RX65	5	—	5	—	—	—	4	—	—	—	—	—	—	7	24/24	1/102
+VE donors/ Total	16/32		13/32		10/31		19/34		18/31		14/32		12/32	24/31	33/33	

[a] Peptide identified from pooling scan (pool 8).
[b] Peptide identified by predictive algorithm.
[c] 12mer peptide identified by decoding pool 30.
[d] 16mer peptide identified by decoding of pool 31.
[e] 12mer peptide identified by decoding pool 33.
[f] 21mer peptide identified by Panina-Bordignon (refs. 4, 11).
[g] Cocktail consists of seven individual peptides each at 1 μM.
[h] 947–967 was shown to be cytotoxic at 10 μM so it was only tested at 1 μM.
[i] Not tested.
[j] Number of wells scored positive ($p < 0.0025$) using 32 replicate wells.
[k] "—" denotes not significantly different from cells alone control ($p > 0.0025$).

that peptides differing in NH_2-terminal position by only one residue would activate different populations of Th cells. If this is the case, the prediction would be that testing smaller numbers of longer peptides could result in failure to detect some epitopes, because peptides with the required NH_2-terminal residues may not be present in the pool. Because overlapping peptides (Tables III to VI) are stimulatory, it is probably not critical to have a particular NH_2-terminal residue to successfully map most epitopes with PBMC. A study on a human tt-specific Th clone (10) found that the NH_2-terminal residue of the minimum stimulatory peptide was the most replaceable amino acid, whereas the COOH-terminal residue could only be changed from I to L, suggesting that the NH_2-termina residue is less significant than implied from peptide isolation studies (15).

APC play a critical role in Ag-stimulated PBMC proliferation assays. Short synthetic peptides can be efficiently presented by a range of APC, including B cells, monocytes and dendritic cells (17). It is known that short peptides can be taken up directly by MHC class II molecules without being processed (18, 19) but the relative significance of this pathway vs an intracellular pathway for peptides presented by APC in PBMC is unknown at this time (20). For longe

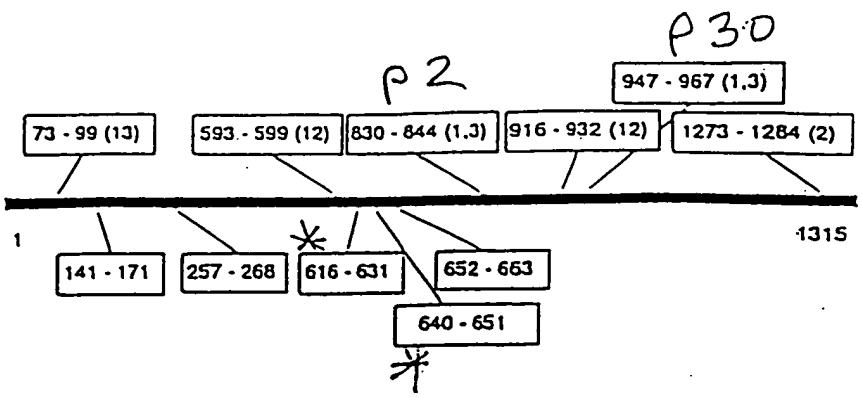

FIGURE 2. A map of the major human Th epitopes of tt. On a linear sc[illegible] the known epitopes are indicated above the line representing the 1315 residues of tt and the newly identified epitopes are indicated below the line.

tides, however, inefficient detection of precursor T cells may be occurring because certain pools of 12mers were stimulatory for PBMC, whereas 31mer peptides spanning the same sequences as the stimulatory pools were not (J.C. Reece et al., manuscript in preparation).

Because we expected the quantitative response of PBMC to be dependent on peptide concentration, we included two dose levels in the survey of seven epitopes (Table VII). We chose to treat significant responses ($p < 0.0025$) at either dose level as representing recognition of an epitope. Although 10 μM often gave higher frequencies of responding wells, there are many instances of the opposite effect, suggesting that this concentration range is a good compromise for most of the peptide/donor combinations.

Better knowledge of the immunodominant and promiscuous epitopes of Ag as determined from unselected Th cells will allow design of reagents for enhancement of immunogenicity of Ag (e.g., vaccines) in humans. Such reagents may be of more general applicability than those established from study of the best-growing clones (21). If antagonistic peptides that have the potential to alleviate autoimmune disease are to be practical (22, 23), the epitopes responsible for disease need to be rapidly located for a spectrum of MHC allotypes without the lengthy and laborious establishment and characterization of clones. This study shows that this can be done for a large Ag.

A cocktail of T cell epitopes may be an effective substitute for whole Ag in diagnostic assays for Th cell function. In the few cases where there was no measurable response to the tt cocktail (Table VII), the responses to individual peptides were seen at only one concentration and were generally low. This cocktail of peptides thus represents a synthetic T cell stimulatory Ag that could be used to standardize T cell proliferation tests on most TT-immune subjects. Serial monitoring of PBMC responses would not be subject to the uncertainty of batch variation in TT or variations in the effective dose of presented peptide.

The identification of the whole spectrum of Th cell epitopes may allow a greater understanding of the basis of epitope selection for MHC class II-restricted epitopes. This may enable accurate prediction of Th cell epitopes from primary sequence data alone.

Acknowledgments

We thank Richard Lauricella, Megan Jay, and the peptide synthesis team for synthesis of the peptides, and the many willing blood donors. We also thank Dr. Gordon Whyte of the Red Cross Blood Bank, Melbourne, for the supply of buffy coats.

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